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Surface-Active Novel Glycolipid and Linked 3-Hydroxy Fatty Acids Produced by *Serratia rubidaea*

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A *Serratia rubidaea* isolate with wetting activity when grown at 30 but not 37°C was examined for the production of specific lipids. Two novel lipids (rubiwettins R1 and RG1) were isolated and shown to be able to lower the surface tension of saline to 26 mN/m. These lipids were located in extracellular vesicles found in a 30°C culture of *S. rubidaea*. Chemical structures of these biosurfactants were determined by degradation product analyses, infrared spectroscopy, mass spectrometry, and proton nuclear magnetic resonance spectroscopy. Rubiwettin R1 was proposed to be a mixture of 3-(3′-hydroxytetradecanoyloxy)decanoate, 3-(3′-hydroxyhexadecenoyloxy)decanoate, and minor molecular isomers. The structure of rubiwettin RG1 was proposed to be β-D-glucopyranosyl 3-(3′-hydroxytetradecanoyloxy)decanoate. The importance of such surface-active exolipids in bacterial occupancy on surfaces was suggested.

Previously, we identified specific aminolipids responsible for prompt spreading of *Serratia marcescens* cultures on various surfaces (1, 12, 14, 16). *S. marcescens* was shown to produce a large amount of such wetting agents (approximately 15% of dried bacterial mass). Three different extracellular aminolipids (W1 from pigmented strain ATCC 13880 or NS 38, W2 from nonpigmented strain NS 25, and W3 from nonpigmented strain NS 45 or NS 50) have been isolated so far. By chemical analyses, these ninhydrin-negative aminolipids were all shown to be cyclodepsipeptides with different amino acids and fatty acids (13) and were named serrawettin (15). It was noteworthy that the production of these serrawettins is markedly influenced by cultivation temperature (12, 14); cultivation at 30°C resulted in massive production of serrawettins, whereas cultivation at 37°C did not. *Serratia rubidaea* is another bacterium that exhibits the wetting activity on cultivation at 30 but not 37°C. Lipids extracted from such a 30°C culture contained specific surface-active lipids (rubiwettins R1 and RG1). By preliminary chemical analyses (13), however, rubiwettins R1 and RG1 were shown to be different from aminolipids. In the study described here, the functions and chemical structures of these novel bacterial lipids were investigated.

MATERIALS AND METHODS

Bacterial strain and growth. *S. rubidaea* ATCC 27593 was described previously (14). The bacteria were grown at 30 or 37°C for 3 days on a peptone glycerol (PG) agar medium containing 5 g of Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 10 ml of glycerol, and 15 g of agar (Eiken, Tokyo, Japan).

Examination of surface activity. The ability to lower surface tension was examined by adding 250 μl of lipid dispersion in 0.15 M NaCl saline (2 mg/ml; prepared by sonication for a few minutes at 90°C) to the surface of 50 ml of saline in a modified Wilhelmy balance (Acoma, Osaka Japan) and measuring the surface tension at 23°C by changing the surface area from 40 cm^2 to 12 cm^2 (10, 12). Wetting activity of a bacterial suspension on water-repelling material was examined as described previously (16).

Preparation of extracellular vesicle fraction. Bacterial mass grown on a PG plate was suspended in 0.15 M NaCl–10 mM potassium-sodium phosphate buffer (PBS; pH 7.4) and centrifuged at 3,500 × *g* for 60 min. The sedimented cells were resuspended in PBS and washed by centrifugation (3,500 × *g*, 20 min) three times. The extracellular vesicles in the supernatant were collected as red amorphous sediment by centrifugation (20,000 × *g*, 3 h).

Preparation of R1 and RG1 lipids. A wet bacterial mass from the surface of PG agar was mixed with 10 volumes of ethanol. After removal of sediment by centrifugation and ethanol by evaporation, the dry material was further extracted with chloroform-methanol (2:1, vol/vol). The extracts were developed on a thin-layer chromatography (TLC) plate of silica gel G (Analtech, Inc., Newark, Del.) in solvent system I (chloroform–methanol–5 M ammonia [80:25:4, vol/vol]). After visualization with iodine vapor, the separated bands were scraped off and extracted with chloroform-methanol (2:1, vol/vol). The extracts were examined on TLC plates with solvent system I, system II (chloroform-methanol-acetone-acetic acid [90:10:6:1, vol/vol]), and system III (chloroform-methanol-acetone [90:10:6, vol/vol]). The extracts giving spots other than R1 or RG1 were developed again on the preparative TLC plate to remove such other lipids.

Degradation and derivatization methods. The lipids were methanolyzed with 5% hydrochloric acid-methanol at 90°C for 15 h in sealed tubes. The resultant fatty acid methyl esters were extracted three times with equal volumes of *n*-hexane. The lower methanol phase was evaporated to dryness after repeated addition of methanol and then examined for polar products. RG1 lipid was alkaline hydrolyzed in 0.2 M sodium hydroxide-chloroform–methanol–water (1:2:0.02, vol/vol) by shaking at 37°C for 1 h. After partition between water and *n*-hexane in the presence of ion-exchange resin (Amberlite, IR-120P), both phases were evaporated to dryness. Methyl esterification was performed by treating the degradation products with an ethereal solution of diazomethane. The mixture was kept at room temperature for 30 min

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and then evaporated under a stream of nitrogen gas. For the preparation of trimethylsilyl (TMS) ether derivatives, the samples were evaporated to dryness, and 50 μl of pyridine and 100 μl of bis(trimethylsilyl)trifluoroacetamide (Nakarai, Kyoto, Japan) were added for each 1-mg sample; the samples were then incubated in a sealed tube at 70°C for 20 min. After the reaction was completed, the solvent and the reaction by-products were coevaporated after the addition of benzene.

All degradation and derivatization products were checked by TLC with solvent system IV (*n*-hexane–diethyl ether [4:1, vol/vol]) for nonpolar products and system V (ethyl acetate-acetic acid-methanol-water [60:15:15:10, vol/vol]) for polar products.

Physicochemical analysis. Gas-liquid chromatography (GLC) was carried out on a Hitachi 663 apparatus with a hydrogen flame ionization detector. The glass-coiled column (1 or 2 m long) was packed with 15% diethylene glycol succinate, 5% SE-30, or 1% OV 101 on Gaschrom Q.

Gas-liquid chromatography-mass spectrometry (GC-MS) was carried out on a Hitachi M-80B double-focusing mass spectrometer with a data processing system (Hitachi M-0101). Conditions for the gas chromatographic column and the inlet were the same as described above. The mass spectra were recorded at an electron energy of 20 eV and accelerating voltage of 3 kV as described previously (9).

Infrared spectra were recorded with a model DS-402G apparatus (Japan Spectroscopic Co., Tokyo, Japan) as a KBr disk.

Scanning of a thin-layer chromatogram was carried out on a Shimadzu CS 900 densitometer. The slit width was 0.3 mm.

Secondary ion mass spectrometry (SIMS) was performed on the mass spectrometer described above, using a beam of xenon ions at an accelerating voltage 8 kV (positive SIMS) or 6 kV (negative SIMS). Glycerol was used as a matrix.

For proton nuclear magnetic resonance (NMR) spectroscopy, about 1 μmol each of R1, RG1, and rhamnolipid [2-O-α-rhamnopyranosyl-α-rhamnopyranosyl 3-(3′-hydroxydecanoyloxy)decanoate; a gift from Y. Ishigami, National Chemical Laboratory for Industry, Tsukuba, Japan] was treated repeatedly with 0.5-ml portions of [^{2}H]methanol, followed by desiccation over P_2O_5 in vacuo to exchange labile protons for deuterons. The thoroughly dried lipids were dissolved in 0.5 ml of [^{2}H]dimethyl sulfoxide-^2H_2O (98:2, vol/vol), and spectra were recorded on a GSX-400 MHz spectrometer (JEOL, Tokyo, Japan) equipped with a tunable probe TH5, PLEXUS data system, and RSX-11M computer system and operated in the Fourier transform mode. One-dimensional spectra were obtained at 30 to 60°C with a sweep width of 4,000 Hz. A total of 65,536 datum points were collected in 9 s of cycling time with irradiation of the water signal. Tetramethylsilane (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was used as an internal standard. Resolution enhancement of one-dimensional spectra was achieved by Gaussian window. Two-dimensional correlation spectroscopy (COSY), relayed COSY, and double-relayed COSY spectra were recorded at 40 and 60 ± 0.5°C with spectral widths of 2,500, 2,000, 1,550, and 750 Hz, and a 2,048- by 1,024-point data matrix was constructed. Special reagents purchased for NMR spectroscopy were as follows: ^2H_2O (99.996%) and [^{2}H]dimethyl sulfoxide (99.95%) from Merck & Co., Inc., Rahway, N.J.); [^{2}H]methanol from Aldrich.

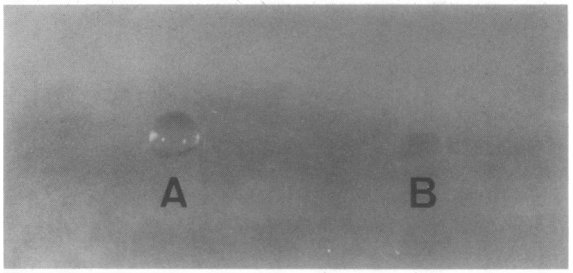

FIG. 1. Wetting activity of *S. rubidaea*. Samples (10 μl) of a bacterial suspension (2 mg [wet weight] suspended in 1 ml of distilled water) of a 37°C culture (A) and a 30°C culture (B; a sinking droplet) were placed on cotton.

RESULTS

Wetting activity and specific lipids of S. rubidaea. *S. rubidaea* ATCC 27593 developed pink wet colonies on a PG plate after incubation at 30°C, whereas colonies after incubation at 37°C were white-pink and dry. Bacterial suspensions made from these two kinds of colonies demonstrated wetting activity to quite different degrees. For example, 10 μl of a 30°C culture suspension sank into water-repelling cotton with ease, whereas a 37°C culture suspension remained as a droplet (Fig. 1). Compositions of lipids extracted from 30 and 37°C cultures were examined and shown to be

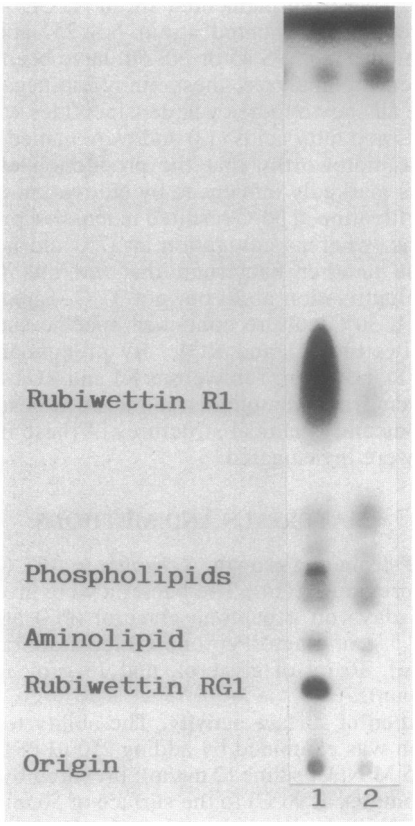

FIG. 2. Thin-layer chromatograms of lipids extracted from a 30°C culture (lane 1) and a 37°C culture (lane 2) of *S. rubidaea*. Each sample (50 μg) was developed with solvent system I. For detection of components, the plate was sprayed with 50% (vol/vol) H_2SO_4 and heated at 200°C for 20 min.

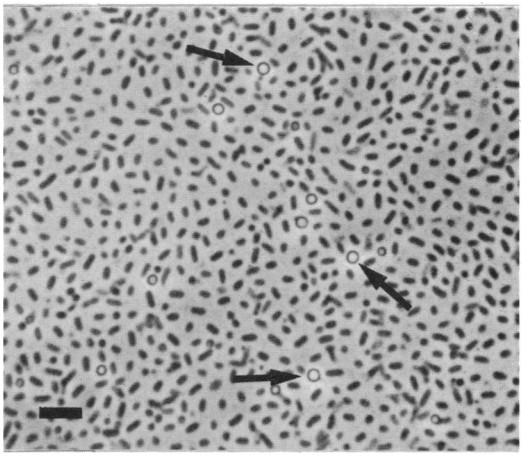

FIG. 3. Phase-contrast micrograph of a 30°C culture of *S. rubidaea*. By examination with a bright-field microscope, the extracellular vesicles (arrows) were shown to be pink (as a result of the presence of prodigiosin), whereas bacterial cells were shown to be colorless. Bar, 5 μm.

markedly different in thin-layer chromatograms (Fig. 2). Two spots (R1 and RG1) that were specific for *S. rubidaea* strains (14, 16) were prominent in lipids from a 30°C culture and negligible in lipids from a 37°C culture. In addition, many extracellular pink vesicles (diameter, 0.2 to ~2 μm) were recognized in a bacterial mass grown on PG agar medium at 30°C (Fig. 3). The vesicles were separated by centrifugation from bacterial cells that were colorless under an optical microscope. TLC of lipids extracted from these separated preparations indicated that R1 and RG1 lipids were exolipids rather than the usual membrane lipids (Fig. 4). Spot R1 (R_f of 0.56 to ~0.59, sometimes split as seen in Fig. 2 and 4) showed no reaction with Dittmer (4), ninhydrin (16), anthrone (17), and α-naphthol (19) reagents. In contrast, spot RG1, with an R_f value (0.10 to ~0.12) close to that of the rhamnolipid spot (data not shown), showed reaction with anthrone and α-naphthol reagents.

Surface activity of R1 and RG1 lipids. R1 and RG1 lipids isolated by preparative TLC each gave a single spot in three different solvent systems, constituted 8.0 and 1.8% of bacterial mass (dry weight), and lowered surface tensions of saline (final concentration, 10 μg/ml of the added lipids) to 25.5 and 25.8 mN/m, respectively (Fig. 5).

Analysis of methanolysates. After acid methanolysis of R1 lipid, no specific products other than small amounts of methyl esters of fatty acids were recognized in a methanol layer by TLC and GLC analyses. On the other hand, methyl glucosides were identified as a sole carbohydrate component in methanolysates of RG1 lipid. The identification was performed by running a methyl glucoside standard (prepared in our laboratory) simultaneously. Methyl esters of fatty acids obtained from R1 and RG1 lipids were examined by GC-MS. These were all shown to be methyl esters of 3-hydroxy fatty acids giving characteristic *m/z* 103 fragment ion or *m/z* 175 fragment ion (from TMS derivatives). Fatty acid compositions of R1 and RG1 lipids determined by GLC of their methanolysates are shown in Table 1.

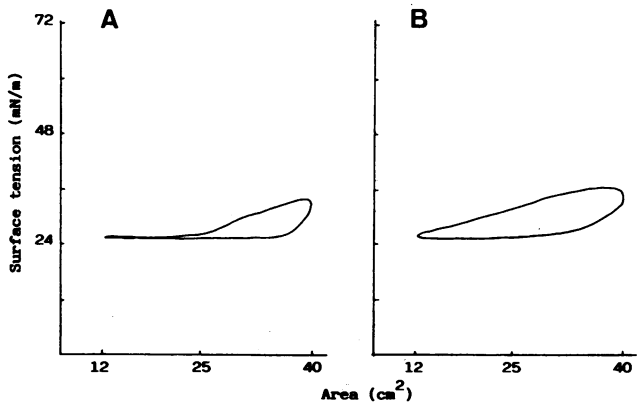

FIG. 5. Surface tension of the changing surface area in the presence of rubiwettins R1 (A) and RG1 (B).

Structure of rubiwettin R1. On TLC with the neutral or alkaline solvent system, R1 lipid showed an elongated spot resembling anionic lipids, whereas in the acidic solvent system the spot was not elongated, suggesting that the compound had a negative charge. Infrared spectroscopy indicated absorption bands of ester linkage (at 1,730 cm^{-1}) and a hydroxyl group (at 3,400 cm^{-1}) in an R1 lipid spectrum. Since the TMS derivative of R1 lipid failed to give peaks on GLC, the lipid was examined directly by positive SIMS analysis. The mass spectrum showed two main ions at *m/z* 545 and 571 (545 + 26), presumably in accordance with the split spot on a TLC plate. After treatment with diazomethane, two main ions at *m/z* 429 (545 − 116) and *m/z* 455 (571 − 116) appeared instead of plus 14 shifted ions. Accordingly, this parallel minus 116 shift was considered to be the result of plus 14 (replacement of −H with $-CH_3$) minus 92 (glycerol) minus 39 (K^+) plus 1 (H^+). The methylated R1

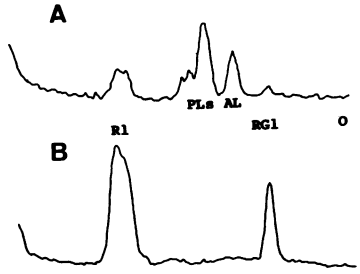

FIG. 4. Density-scanned thin-layer chromatograms of lipids extracted from separated fractions. (A) Bacterial cells; (B) extracellular vesicles. TLC was carried out as described in the legend to Fig. 2. R1, Rubiwettin R1; PLs, phospholipids; AL, L-lysine-containing aminolipid; RG1, rubiwettin RG1, O, origin.

TABLE 1. Fatty acid compositions of rubiwettins R1 and RG1

Fatty acid	Composition (%)	
	R1	RG1
3-OH $C_{10:0}$	18.4	32.5
3-OH $C_{12:0}$	1.7	0.7
3-OH $C_{12:1}$	3.5	0.6
3-OH $C_{14:0}$	26.4	57.4
3-OH $C_{14:1}$	5.7	6.3
3-OH $C_{14:0,br}$	3.4	ND[a]
3-OH $C_{16:0}$	7.5	0.7
3-OH $C_{16:1}$	30.3	1.9
3-OH $C_{16:0,br}$	1.1	ND

[a] ND, Not detectable.

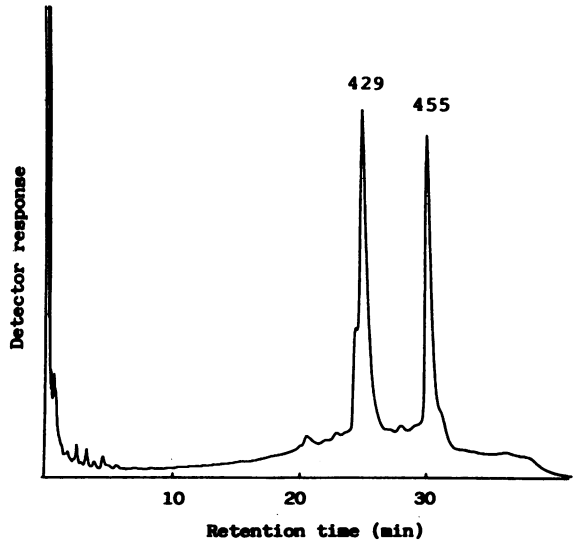

FIG. 6. Gas chromatogram of methylated R1 lipid (5% SE-30, 200 to 270°C). The mass number in each peak was determined by GC-MS.

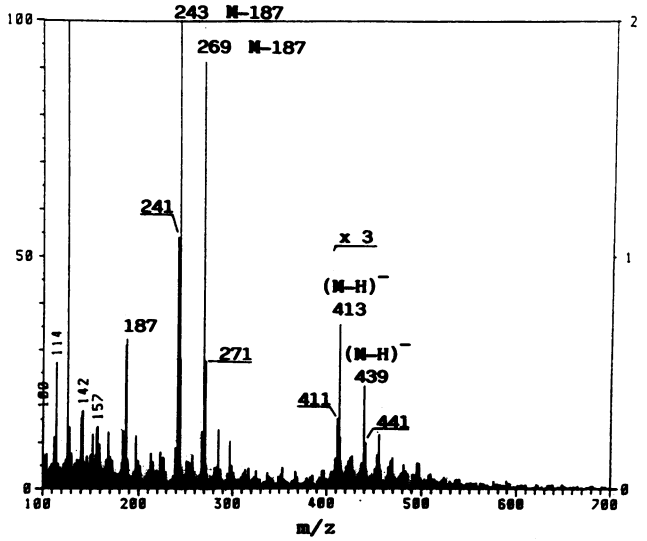

FIG. 8. Negative secondary ion mass spectrum of rubiwettin R1. Ions with underlined mass number correspond to minor fatty acid isomers. Loss of the C_{10} carboxyl moiety resulted in each minus 187 ion.

lipid gave two main peaks on GLC (Fig. 6), which were examined for molecular and fragment ions by GC-MS. Thus, the two corresponding ions of *m/z* 429 and 455 were identified as methyl esters of 3-(3′-hydroxytetradecanoyloxy)decanoic acid (Fig. 7) and 3-(3′-hydroxyhexadecenoyloxy)decanoic acid, respectively. Actually, R1 lipid itself was shown to exhibit $(M - H)^-$ ions at *m/z* 413 and 439 in negative SIMS (Fig. 8). The presence of a cluster of ions at (M − 187) was noteworthy. A hydroxyl group of 3-hydroxydecanoic acid seems to be commonly acylated in a series of R1 isomers. As summarized in Fig. 12A, rubiwettin R1 is proposed to be a mixture of linked 3-hydroxy fatty acids, i.e., 3(3′-hydroxy) hydroxy fatty acids.

Structure of the aglycon moiety of rubiwettin RG1. After mild alkaline hydrolysis of RG1 lipid, a hexane-soluble product showing a behavior on TLC (R_f value and reactivity to chromogenic spray reagents) similar to that of R1 lipid was obtained. An infrared spectrum of the product was also similar, especially with respect to absorption bands at 1,730 and 3,400 cm^{-1}. A major diazomethane-treated derivative of this product was shown to be a methyl ester of 3-(3′-hydroxytetradecanoyloxy)decanoic acid by GC-MS analysis (Fig. 9). Consistent with these data, acid hydrolysis of this lipophilic alkaline hydrolysate resulted in two main products, 3-hydroxydecanoic acid and tetradecenoic acid (dehydration product of 3-hydroxytetradecanoic acid). Thus, the main aglycon component forming rubiwettin RG1 was considered to be 3-(3′-hydroxytetradecanoyloxy)decanoic acid which is one of the major linked 3-hydroxy fatty acids composing rubiwettin R1, as shown above.

Mass spectrometry of rubiwettin RG1. In Fig. 10, a mass spectrum of RG1 lipid examined by positive SIMS is shown. Protonated molecular ion (MH^+) at *m/z* 577, pseudomolecular ion $(M + Na)^+$ at *m/z* 599, and fragment ion at *m/z* 415 (presumably a derivative of the aglycon moiety) are evident. Addition of NaCl to the sample resulted in no shift of 599 ion. Thus, a major RG1 lipid seemed to be a condensation product of equimolar amounts of glucose and 3-(3′hydroxytetradecanoyloxy)decanoic acid. Since RG1 lipid was meth-

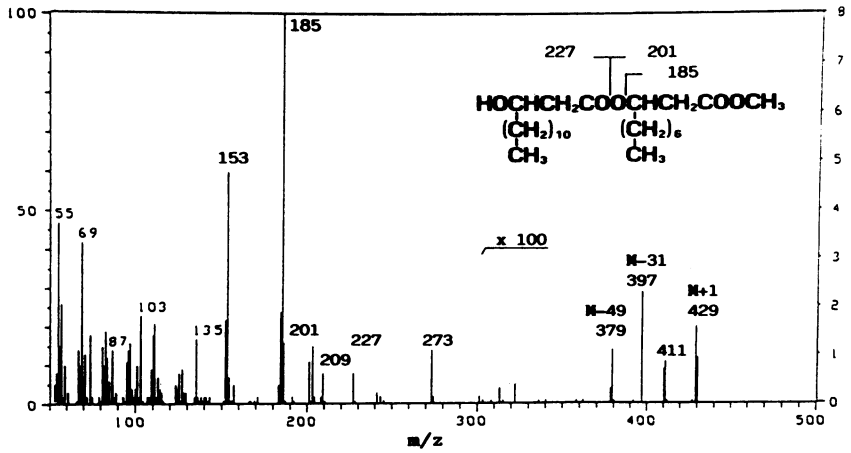

FIG. 7. Mass spectrum of methylated R1 (peak 429 in Fig. 6). In addition to ions (M+1 and M-H_2O and/or -OCH_3), ions indicating the presence of the 3-hydroxytetradecanoyl moiety (227, 209) are evident.

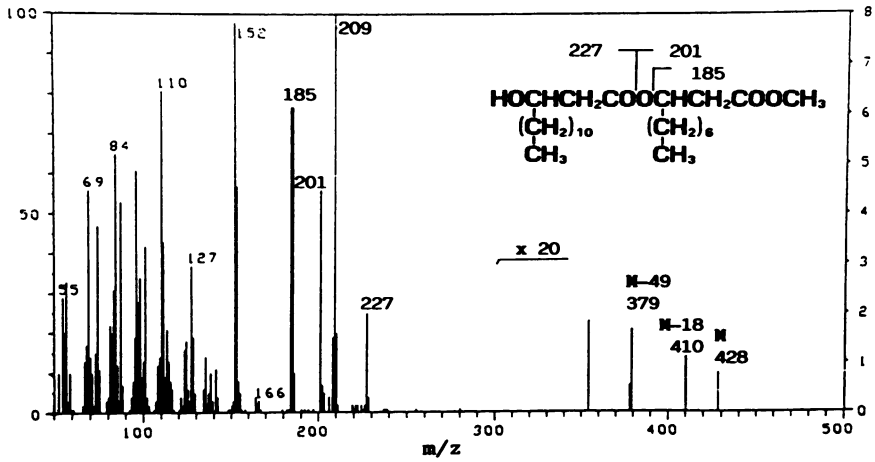

FIG. 9. Mass spectrum of a methylated hexane-soluble alkaline hydrolysate of rubiwettin RG1.

ylated with ease by the diazomethane treatment and gave a mass spectrum showing plus 14 mass unit shifted ions from 599 and 415 ions seen in Fig. 10, the aglycon moiety of RG1 lipid must have a free carboxyl group.

Proton NMR. The ^1H assignments described in Fig. 11 and Table 2 were based on the results obtained by two-dimensional NMR spectra (Fig. 11b). A proton-proton shift correlation of RG1 lipid afforded all of the connectivities needed for the assignment of all protons of glucose and protons at carbons 2, 3, and 4 of fatty acids. The series of ring protons H-1 to H-6 of glucose were neatly separated from each other, and the coupling could successively be assigned starting from the anomeric proton at the lowest magnetic field (4.195 ppm; Fig. 11b). The coupling constant ($^3J_{1,2}$) of 7.8 Hz unequivocally indicated that the anomeric configuration of glucose is β. By comparison of the chemical shift (Table 2) with those of glucosylceramide (6), which also has a terminal glucose, significant lower field shifts due to attachment of glycoside or ester were not found.

A multiplet (RA-3) observed at 5.080 ppm, coupled with two double doublets at 2.441 (RA-2b) and 2.382 ppm (RA-2a) as well as a double doublet at 1.55 ppm (RA-4), was assigned to the proton at acylated carbon 3 of 3-hydroxy fatty acid. The chemical shift of RA-3 was close to the protons at carbon 3 of acylated 3-hydroxy fatty acids of R1 lipid (5.090 ppm) and an acylated oligosaccharide fragment derived from *Escherichia coli* lipid A (5.10 ppm) (7). The chemical shift of a multiplet at 3.916 ppm (RS-3) assigned as the proton at carbon 3 of glucosylated 3-hydroxytetradecanoic acid was close to the corresponding signal of rhamnolipid at 3.884 ppm, whereas the proton at carbon 3 of nonglucosylated 3-hydroxy fatty acids of R1 lipid gave a signal at 3.780 ppm. RS-3 was coupled with two double doublets at 2.732 (RS-2b) and 2.353 ppm (RS-2a) and a multiplet at 1.45 ppm (RS-4). The results of the irradiation and integration of RA-3, RS-3, etc., consistently supported this identification and substantiated the presence of equimolar amounts of 3-acylated and 3-glucosylated fatty acids. A down-field multiplet at 5.323 ppm, coupled with methylenes (1.984 ppm) adjacent to *cis*-olefinic methine protons, was due to *cis* double bonds in 3-hydroxytetradecenoic acid and 3-hydroxyhexadecenoic acid (minor variants of the fatty acids as shown in Table 1). A proposed structure of the major component of rubiwettin RG1 is shown in Fig. 12B.

DISCUSSION

In this present study, rubiwettin R1 is proposed to be a mixture of linked 3-hydroxy fatty acids (Fig. 12A). The

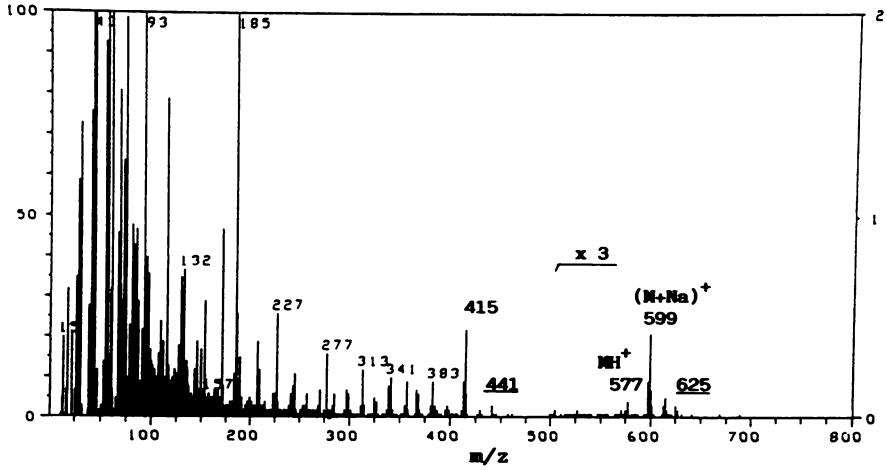

FIG. 10. Positive secondary ion mass spectrum of rubiwettin RG1. Ions with underlined mass number correspond to a minor fatty acid isomer containing 3-hydroxyhexadecenoic acid.

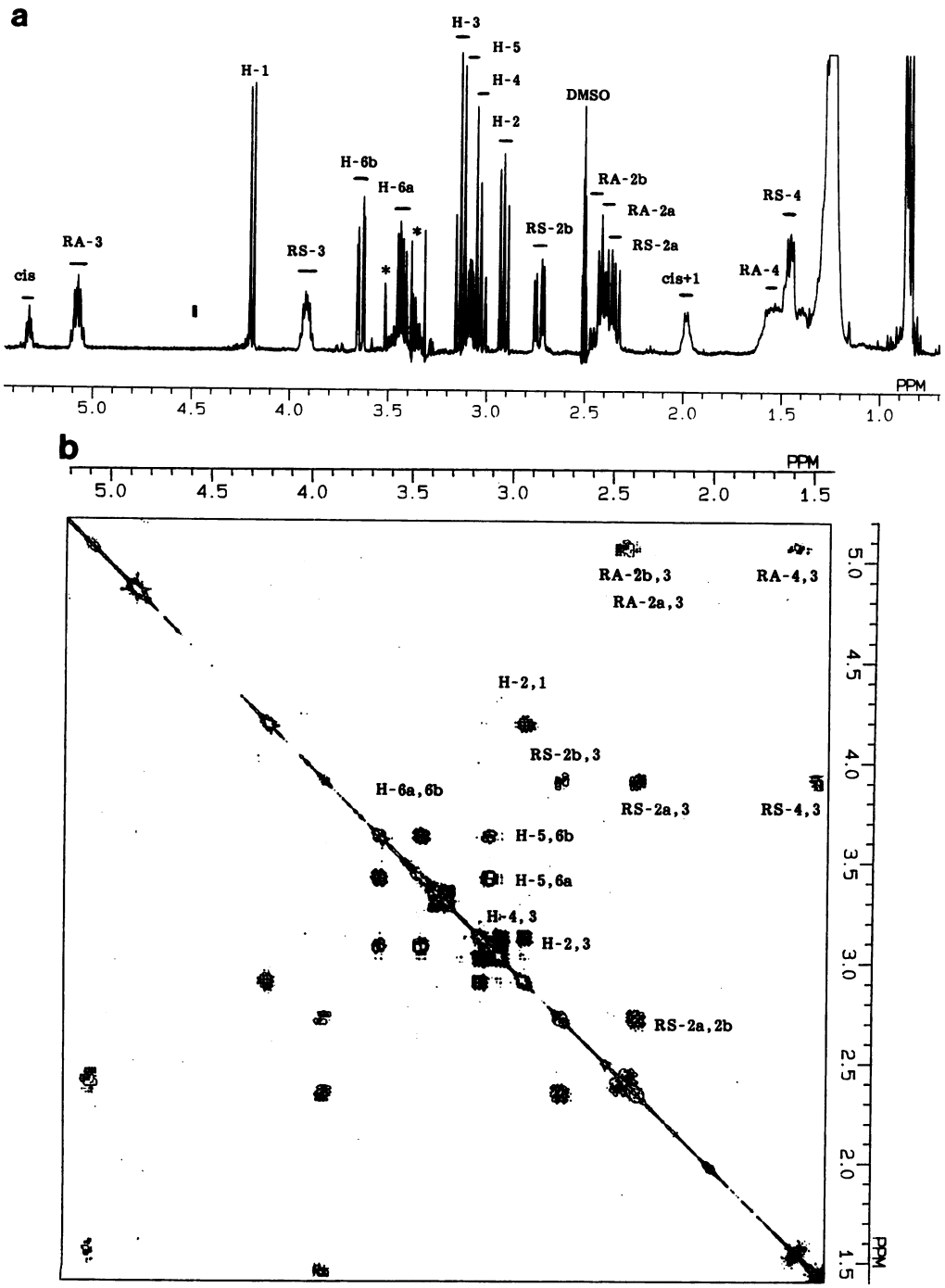

FIG. 11. Proton NMR spectra of rubiwettin RG1. (a) One-dimensional spectrum. *, Unidentified contaminants; cis, olefinic protons of *cis* double bond in fatty acids; cis + 1, methylene protons adjacent to *cis* double bonds. For other symbols, see the text and Table 2, footnote *a*. (b) Two-dimensional COSY spectrum. The cross-peaks between two reasonances are indicated with symbols separated with a comma.

major components were 3-(3′-hydroxytetradecanoyloxy)decanoate and 3-(3′-hydroxyhexadecenoyloxy)decanoate. In addition, the presence of minor variants such as 3-(3′-hydroxytetradecenoyloxy)decanoate and 3-(3′-hydroxyhexadecanoyloxy)decanoate was suggested (Table 1 and Fig. 8). Rubiwettin RG1 was β-D-glucopyranosyl 3-(3′-hydroxytetradecanoyloxy)decanoate and contained minor fatty acid isomers consisting of 3-(3′-hydroxytetradecenoyloxy)decanoate or 3-(3′-hydroxyhexadecenoyloxy)decanoate (Fig. 12B). Thus, R1 and RG1 exolipids produced in parallel by *S. rubidaea* were indicated as novel types of amphipathic lipids.

We have isolated spontaneous mutants defective in production of rubiwettin RG1 by the new method of direct colony TLC (16). These mutants, e.g., strain WH-05 described in a previous report (16), demonstrated markedly decreased wetting activity on water-repelling material despite production of rubiwettin R1 (T. Matsuyama et al., unpublished results). Therefore, rubiwettin RG1 may be a

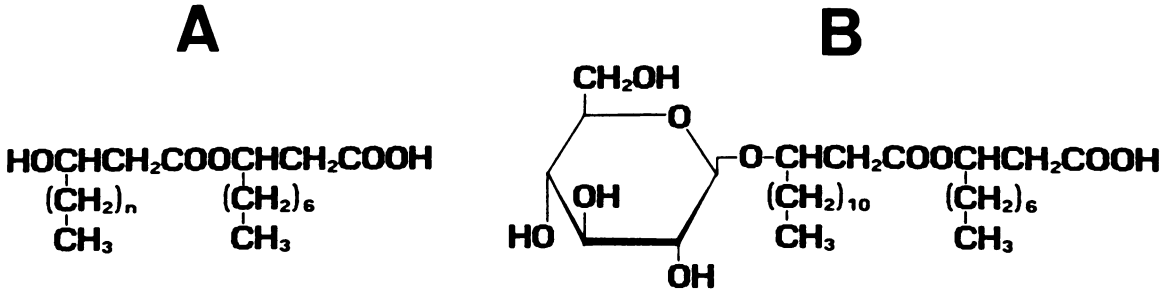

FIG. 12. Proposed structures of rubiwettins R1 (A) and RG1 (B). The generalized structure of R1 (n = 8, 10, 12, saturated or monounsaturated) and the structure of a major isomer of RG1 are shown.

physiologically important final product. By unknown specific condensation reactions, 3-(3'-hydroxytetradecanoyloxy) decanoate seemed to be preferentially glucosylated.

In a study of enzymatic rhamnolipid synthesis by extracts of *Pseudomonas aeruginosa* (2), 3-(3'-hydroxydecanoyloxy) decanoate (not a natural product; prepared by partial acid hydrolyis of rhamnolipid) was shown to be a good acceptor of rhamnose. R1 lipid, however, may not be a simple RG1 precursor that was unusually overproduced in the strain used in the study described here. We have previously reported marked production of R1 lipid by another *S. rubidaea* strain, CDC 299-72 (previously *Serratia marinorubra*) (14). The virtual absence of 3-(3'-hydroxyhexadecenoyloxy)decanoate from RG1 is also noteworthy. The strong surface activity of the isolated R1 (Fig. 5A) and a large amount of production in response to culture conditions suggest some unknown functions of this unique exolipid.

Rubiwettin RG1 is different from most bacterial glycolipids such as acylglycoses or glycosyldiacylglycerols (3, 18). As in the rhamnolipids (5, 8), a linked hydroxy fatty acid is not bound to a sugar moiety by an acyl bond. A glycosidic bond with a hydroxyl group of the fatty acid is formed. Rubiwettin RG1 is an exolipid and in this respect is also similar to rhamnolipids and dissimilar from most other glycolipids. Taxonomically, *S. rubidaea* is quite remote from rhamnolipid-producing *P. aeruginosa*. The questions of whether both unique exolipids have common physiological functions and both species share a common habitat in nature remain to be solved. Since the physiological functions of lipids are largely obscure, it is generally difficult to find novel lipids by detecting their specific activities. Extensive chemical surveys on bacterial lipids may disclose the presence of rhamnolipidlike exolipids among other bacterial species. As indicated here, however, culture conditions are sometimes critical. Temperature-dependent production of exolipids has been also observed in the studies on *S. marcescens* lipids. The production of serawettins W1, W2, and W3, which have been identified as cyclodepsipeptides (13), is a typical example.

We noted peculiar spreading growth of *S. marcescens* on Davis or Vogel-Bonner minimal solid agar incubated at 30°C (temperature for production of serrawettins) (15). The resultant giant colony (diameter, 60 to ~70 mm after 3 weeks of incubation) had an irregular fiord-like outline. By examining a statistical self-similarity of the outlines as described by Mandelbrot (11), we showed that the outlines of the spreading *S. marcescens* colonies are fractal (fractal dimension, 1.4 to ~1.6) (15). Such fractality promises an extremely long front line to the growing cell mass. Consequently, the cell mass will make efficient interactions with surrounding environments and expand successively on the surface. To elucidate the role of surface-active lipids in such flagellum-independent spreading growth, we isolated serrawettinless mutants and then revealed the promotive role of serrawettins in the fractal spreading growth of *S. marcescens* (15). We also examined the fractal spreading growth exhibited by *S. rubidaea*. Thus far, we have observed retarded spreading growth of mutants defective in production of rubiwettin RG1. Precise experimental studies will be done by preparing a series of mutants lacking either or both rubiwettins R1 and RG1.

TABLE 2. ^{1}H chemical shifts and coupling constants of rubiwettin RG1 at 40°C[a]

Proton	Chemical shift (ppm)	Coupling constant (Hz)
RS-2a	2.353	−14.9[b] (RS-2a,2b)
RS-2b	2.732	5.1 (RS-2b,3)
RA-2a	2.382	−15.6[c] (RA-2a,2b)
RA-2b	2.441	7.7 (RA-2b,3)
RS-3	3.916	8.4 (RS-2a,3)
RA-3	5.080	6.0 (RA-2a,3)
RS-4	1.45	7.1[b] (RS-4,5)
RA-4	1.55	
cis	5.323	
cis + 1	1.984	
Glc H-1	4.195	7.8 (1,2)
Glc H-2	2.911	8.8 (2,3)
Glc H-3	3.134	8.7 (3,4)
Glc H-4	3.029	8.8 (4,5)
Glc H-5	3.088	5.5 (5,6a)
Glc H-6a	3.431	−11.5 (6a,6b)
Glc H-6b	3.640	2.2 (5,6b)

[a] RS, Glucosylated 3-hydroxy fatty acid moiety; RA, acylated 3-hydroxy fatty acid moiety.
[b] Obtained by irradiation of RS-3.
[c] Obtained by irradiation of RA-3.

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